Galectin-1 Accelerates Wound Healing by Regulating the Neuropilin-1/Smad3/NOX4 Pathway and ROS Production in Myofibroblasts

Yueh-Te Lin[1], Jhih-Sian Chen[2], Ming-Heng Wu[3], I-Shan Hsieh[2], Chen-Hsien Liang[1], Cheng-Lung Hsu[4], Tse-Ming Hong[1,5] and Yuh-Ling Chen[1,2]

Myofibroblasts have a key role in wound healing by secreting growth factors and chemoattractants to create new substrates and proteins in the extracellular matrix. We have found that galectin-1, a β-galactose-binding lectin involved in many physiological functions, induces myofibroblast activation; however, the mechanism remains unclear. Here, we reveal that galectin-1-null (*Lgals1*$^{-/-}$) mice exhibited a delayed cutaneous wound healing response. Galectin-1 induced myofibroblast activation, migration, and proliferation by triggering intracellular reactive oxygen species (ROS) production. A ROS-producing protein, NADPH oxidase 4 (NOX4), was upregulated by galectin-1 through the neuropilin-1/Smad3 signaling pathway in myofibroblasts. Subcutaneous injection of galectin-1 into wound areas accelerated the healing of general and pathological (streptozotocin-induced diabetes mellitus) wounds and decreased the mortality of diabetic mice with skin wounds. These findings indicate that galectin-1 is a key regulator of wound repair that has therapeutic potential for pathological or imperfect wound healing.

Journal of Investigative Dermatology (2015) **135,** 258–268; doi:10.1038/jid.2014.288; published online 14 August 2014

INTRODUCTION

Wound healing is a complicated physiological process that includes hemostasis, inflammation, proliferation, and a remodeling phase (Singer and Clark, 1999; Diegelmann and Evans, 2004; Velnar *et al.,* 2009). Numerous cell signaling events are required for one or more phases and regulate this highly controlled repair process (Diegelmann and Evans, 2004; Clark *et al.,* 2007). In pathologic conditions such as diabetes mellitus, ischemia and increased pressure can result in chronic and nonhealing wounds (Guo and Dipietro, 2010). Approximately 3 million people in the United States and 2% of the population of industrialized countries suffer from nonhealing wounds every year (Menke *et al.,* 2007; Welt *et al.,* 2009). Furthermore, the number of patients with chronic wound healing is expected to increase because of the increase in the elderly population over the next 20 years. Therefore, a greater understanding of the disease pathology and identification of rapid and effective therapies for impaired wound healing are urgently needed.

During wound healing, fibroblasts are activated to become myofibroblasts that migrate to wound areas. Myofibroblasts have key roles in wound healing, including growth factor secretion, extracellular matrix synthesis, and angiogenesis (Tomasek *et al.,* 2002; Sarrazy *et al.,* 2011). In nonhealing and chronic wounds, fibroblasts display cellular characteristics of premature senescence (Harding *et al.,* 2005; Clark, 2008), increased apoptosis (Graves *et al.,* 2006), disturbed proliferation (Loots *et al.,* 1999), and little response to stimulation (Lerman *et al.,* 2003). Recent studies have suggested that targeting myofibroblast differentiation may offer a promising therapeutic strategy for the treatment of improper or impaired wound healing (Sampson *et al.,* 2012; Lai *et al.,* 2013).

Galectin-1 is a prototypic member of the galectin family that binds β-galactoside and is differentially expressed by various normal and pathological tissues. Galectin-1 regulates a wide range of biological functions such as cell growth, cell adhesion, metastasis, angiogenesis, neuron development, cardiovascular disease, and immunomodulation (Thijssen *et al.,* 2006; Salatino *et al.,* 2008; Wu *et al.,* 2009; Sakaguchi *et al.,* 2011; Smetana *et al.,* 2013; Al-Salam and Hashmi, 2014). Previous studies have indicated that galectin-1

[1]*Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan, Taiwan;* [2]*Institute of Oral Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan;* [3]*Institute for Translational Medicine, Taipei Medical University, Taipei, Taiwan;* [4]*Department of Internal Medicine, Division of Hematology and Oncology, Chang Gung Memorial Hospital, Taoyuan, Taiwan and* [5]*Graduate Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan*

Correspondence: Yuh-Ling Chen or Tse-Ming Hong, Institute of Oral Medicine, College of Medicine, National Cheng Kung University, No. 1 University Road, Tainan, 70101, Taiwan.
E-mail: yuhling@mail.ncku.edu.tw or tmhong@mail.ncku.edu.tw

Abbreviations: HGF, human gingival fibroblast; H_2O_2, hydrogen peroxide; NOX4, NADPH oxidase 4; NRP1, neuropilin-1; O_2^-, superoxide; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; TGF-β, transforming growth factor-β; α-SMA, α-smooth muscle actin; WT, wild type

Received 12 March 2014; revised 20 May 2014; accepted 4 June 2014; accepted article preview online 9 July 2014; published online 14 August 2014

is enriched in cancer-associated fibroblasts and promotes growth and invasion of adjacent cancer cells (Wu *et al.,* 2011; Xue *et al.,* 2011). Galectin-1 has been associated with myofibroblast differentiation in murine dermal fibroblasts, and treatment with galectin-1 was found to promote myofibroblast activation (Goldring *et al.,* 2002). Cytokine production and proliferation in pancreatic stellate cells or transdifferentiation of hepatic stellate cells are induced by high expression of galectin-1 (Maeda *et al.,* 2003; Maeda *et al.,* 2004; Masamune *et al.,* 2006). Moreover, galectins have been reported to be regulated during wound healing *in vitro* and *in vivo* (Klíma *et al.*, 2009; Dvořánková *et al.,* 2011; Gál *et al.*, 2011). Although galectin-1 is an important mediator of myofibroblast activation, the mechanism and regulation of galectin-1-mediated myofibroblast activation and wound healing are still unclear. Therefore, we hypothesized that galectin-1 participates in wound healing by inducing myofibroblast activation. In this study, we used galectin-1 knockout (*Lgals1*$^{-/-}$) mice to examine the role of galectin-1 in cutaneous wound healing and address the molecular mechanism underlying galectin-1-induced fibroblast activation. The potential therapeutic effect of galectin-1 on physiological and pathological wound healing was also evaluated.

RESULTS

Lgals1$^{-/-}$ mice have delayed cutaneous wound healing compared with wild-type mice

To test our hypothesis that galectin-1 is involved in the regulation of wound healing *in vivo*, we used a skin wound healing model and *Lgals1*$^{-/-}$ mice to examine the effect of galectin-1 on skin wound healing. After dermal excision, wound areas were measured every other day for 8 days. We found that *Lgals1*$^{-/-}$ mice had bloody wounds and poor scar formation, and they exhibited signs of inflammation at day 2 and delayed wound closure at day 5 (Figure 1a). By day 7, ~90% of the wound was closed in wild-type (WT) mice, compared with 50% in *Lgals1*$^{-/-}$ mice (Supplementary Figure S1a online). Quantification of the wound area by ImageJ software (National Institutes of Health, Bethesda, MD) for 8 consecutive days showed that the rate of wound healing was significantly decreased in *Lgals1*$^{-/-}$ mice relative to WT mice (Figure 1b). Reepithelization and collagen deposition in *Lgals1*$^{-/-}$ mouse wounds measured by hematoxylin and eosin and Masson's trichrome staining were also less than those in WT mice (Figure 1c and Supplementary Figure S1b online), especially in *Lgals1*$^{-/-}$ mouse 3. However, the sublayers of the skin exhibited no significant differences between WT and *Lgals1*$^{-/-}$ mice (Supplementary Figure S1c online).

Collectively, these data suggest that *Lgals1*$^{-/-}$ mice have poor wound healing ability compared with WT mice. Total protein from the skin and wound areas of the mice was collected to detect the expression of galectin-1, α-smooth muscle actin (α-SMA), and fibronectin by western blotting. In WT mice, galectin-1 was upregulated in the wound areas (W1–W3) compared with normal skin (N1–N3), and expression of α-SMA (a major myofibroblast activation marker, Darby *et al.,* 1990) and fibronectin was also increased (Figure 1d). The α-SMA level in the wound areas was noticeably lower in *Lgals1*$^{-/-}$ mice than in WT mice. Immunohistochemistry revealed that more myofibroblasts were migrating toward the wound areas in WT mice than in *Lgals1*$^{-/-}$ mice at day 3 (Supplementary Figure S1d online). This result suggests that galectin-1 facilitates myofibroblast activation and wound healing *in vivo*.

Galectin-1 has a pleiotropic role in myofibroblast activation

To examine the functional role of galectin-1 in fibroblasts, we treated human gingival fibroblasts (HGFs) with active and heat-inactivated galectin-1. Treatment of HGFs with active galectin-1 increased the expression of α-SMA relative to treatment with denatured galectin-1 (Figure 2a). Consistently, knockdown of galectin-1 by short hairpin RNA (shGal-1) in activated fibroblasts, cancer-associated fibroblasts, and skin myofibroblasts (CCD966-SKs) was accompanied by decreased expression of α-SMA, fibronectin, and collagen IA (Figure 2b and c). The migration and proliferation of myofibroblasts were significantly suppressed in shGal-1-myofibroblasts (Figure 2d and e). Taken together, these results suggest that galectin-1 has a key role in myofibroblast activation.

Galectin-1 induces ROS production in myofibroblasts and an animal model

Several studies have indicated that reactive oxygen species (ROS) generation is a key step during myofibroblast activation (Cucoranu *et al.,* 2005; Amara *et al.,* 2010; Bondi *et al.,* 2010; Sampson *et al.,* 2011). In light of previous findings that galectin-1 induced ROS generation in neutrophils (Almkvist *et al.,* 2002), we were interested in determining whether ROS would be generated in myofibroblasts treated with galectin-1. First, we detected superoxide (O_2^-) and hydrogen peroxide (H_2O_2) levels in several types of fibroblasts using lucigenin and 2′,7′-dichlorodihydrofluorescein diacetate, respectively. We found that O_2^- and H_2O_2 levels in myofibroblasts were tightly associated with galectin-1 expression (Figure 3a and Supplementary Figure S2a and b online). ROS have been reported to induce corneal wound healing *in vivo* (Huo *et al.,* 2009) and provide detoxification for wound healing (auf dem Keller *et al.,* 2006). We therefore also measured the ROS levels in normal skin and wounds in WT and *Lgals1*$^{-/-}$ mice. We found that *Lgals1*$^{-/-}$ mice had lower ROS generation in normal skin and wounds compared with WT mice (Figure 3b). Importantly, in the presence of the ROS scavenger *N*-acetyl-L-cysteine, α-SMA expression and the proliferation and migration of myofibroblasts decreased in a concentration-dependent manner (Figure 3c–e). Moreover, treatment of H_2O_2 (0–60 μmol l^{-1}) for 2 days dose-dependently stimulated cell proliferation of shGal-1-myofibroblasts (Supplementary Figure S2c online). These results indicate that ROS generation is essential for galectin-1-induced myofibroblast activation.

Galectin-1 induces ROS generation in myofibroblasts by regulating NOX4

Several studies have indicated that the NAD(P)H oxidase family has an important role in ROS production in various

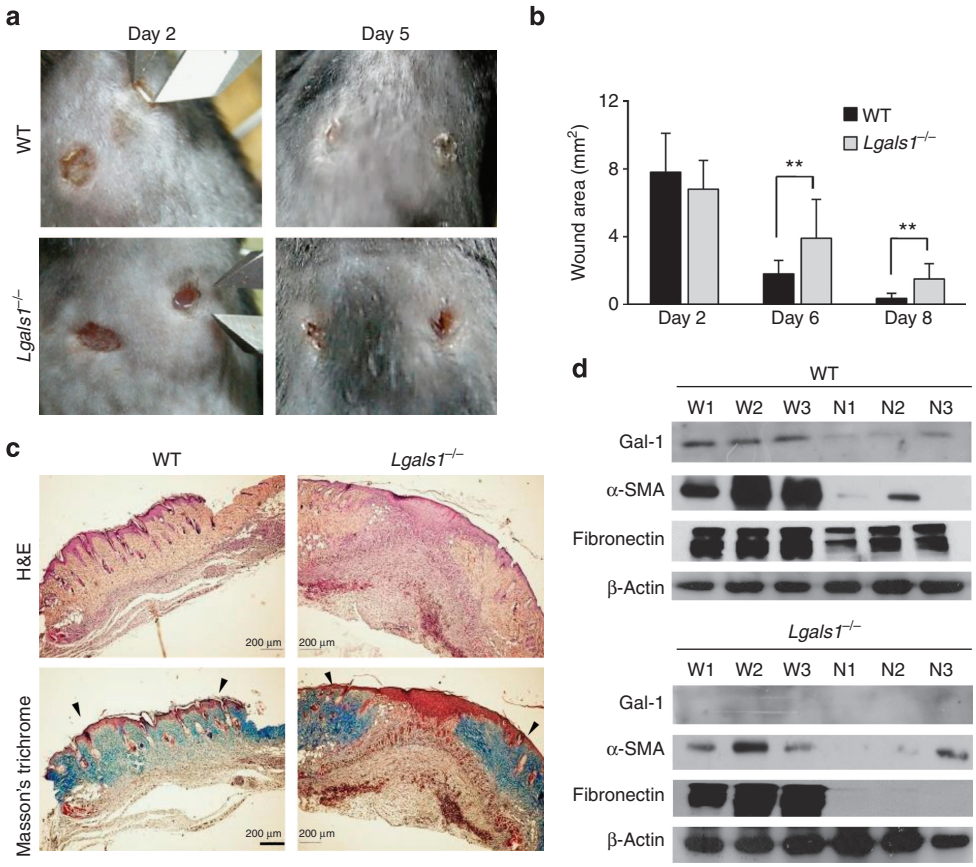

Figure 1. *Lgals1*$^{-/-}$ mice have delayed wound healing compared with wild-type (WT) mice. (**a**) Representative macroscopic images of wounds in WT and *Lgals1*$^{-/-}$ mice at 2 and 5 days after wounding show delayed healing in *Lgals1*$^{-/-}$ wounds. (**b**) The areas of the wounds were quantified by ImageJ (NIH) over multiple time points (four wounds/mouse, $n = 10$–15 mice per group). Values represent the mean ± SD. $^{**}P \leqslant 0.01$. (**c**) Representative hematoxylin and eosin and Masson's trichrome staining in the WT and *Lgals1*$^{-/-}$ wounds at day 8. Arrowheads indicate wound margins. Scale bars = 200 μm. (**d**) Western blotting of galectin-1, α-smooth muscle actin (α-SMA), and fibronectin in three randomly chosen excisional wounds (W1–W3) and normal skin (N1–N3) from WT and *Lgals1*$^{-/-}$ mice. β-Actin was used as a loading control.

cell types (Cucoranu *et al.,* 2005; Bedard and Krause, 2007; Hecker *et al.,* 2009). In fibroblasts, NADPH oxidase 4 (NOX4) is a major ROS-generating protein and regulates cell hemostasis. Therefore, we hypothesized that galectin-1 induces ROS production in fibroblasts by regulating the expression of NOX4. We found that expression of NOX4 and NADPH oxidase activity were significantly decreased in Gal-1-silenced oral and skin myofibroblasts (Figure 4a and Supplementary Figures S2d and S3 online). Moreover, we found that silencing of NOX4 using NOX4-specific short hairpin RNA apparently decreased the expression of α-SMA in myofibroblasts (Figure 4b). Suppression of endogenous NOX4 also resulted in a decrease in O_2^- and H_2O_2 generation (Supplementary Figure S2e and f online). Furthermore, we found that NOX4 knockdown effectively inhibited the proliferation and migration of oral and skin myofibroblasts (Figure 4c and d). Immunofluorescence analysis revealed that galectin-1, NOX4, and α-SMA colocalized at the site of injury and fewer myofibroblasts were present in *Lgals1*$^{-/-}$ mice (Figure 4e and Supplementary Figure S4 online). Collectively, these results indicate that NOX4 mediates galectin-1-induced ROS production and myofibroblast activation.

Galectin-1 induces myofibroblast activation through neuropilin-1 binding and the Smad3/NOX4 pathway

We next investigated the possible mechanism of galectin-1-induced NOX4 expression. Our previous studies showed that galectin-1 can bind neuropilin-1 (NRP1) on vascular endothelial cells to regulate angiogenesis (Hsieh *et al.,* 2008). Recent studies have indicated that NRP1 promotes myofibroblast activation by enhancing Smad pathway signaling in a variety of physiological and disease conditions (Cao *et al.,* 2010a, b). Hence, we first examined whether galectin-1 binds NRP1 on fibroblasts and induces Smad3 phosphorylation. In a flow cytometric analysis, FITC-conjugated galectin-1-binding HGFs were abolished by silencing of NRP1 (Figure 5a), indicating that NRP1 is a major binding site for galectin-1 in fibroblasts. HGFs treated with galectin-1 exhibited an increase in Smad3 phosphorylation (Ser423/425) (Supplementary Figure S5a online). Furthermore, knockdown of NRP1 in myofibroblasts suppressed Smad3 activation, NOX4 expression, ROS production, and concomitant myofibroblast activation (Figure 5b and c, and Supplementary Figure S5b online).

Treatment with lactose, a galactoside competitor for galectin-1 binding, attenuated Smad3/NOX4/α-SMA pathway

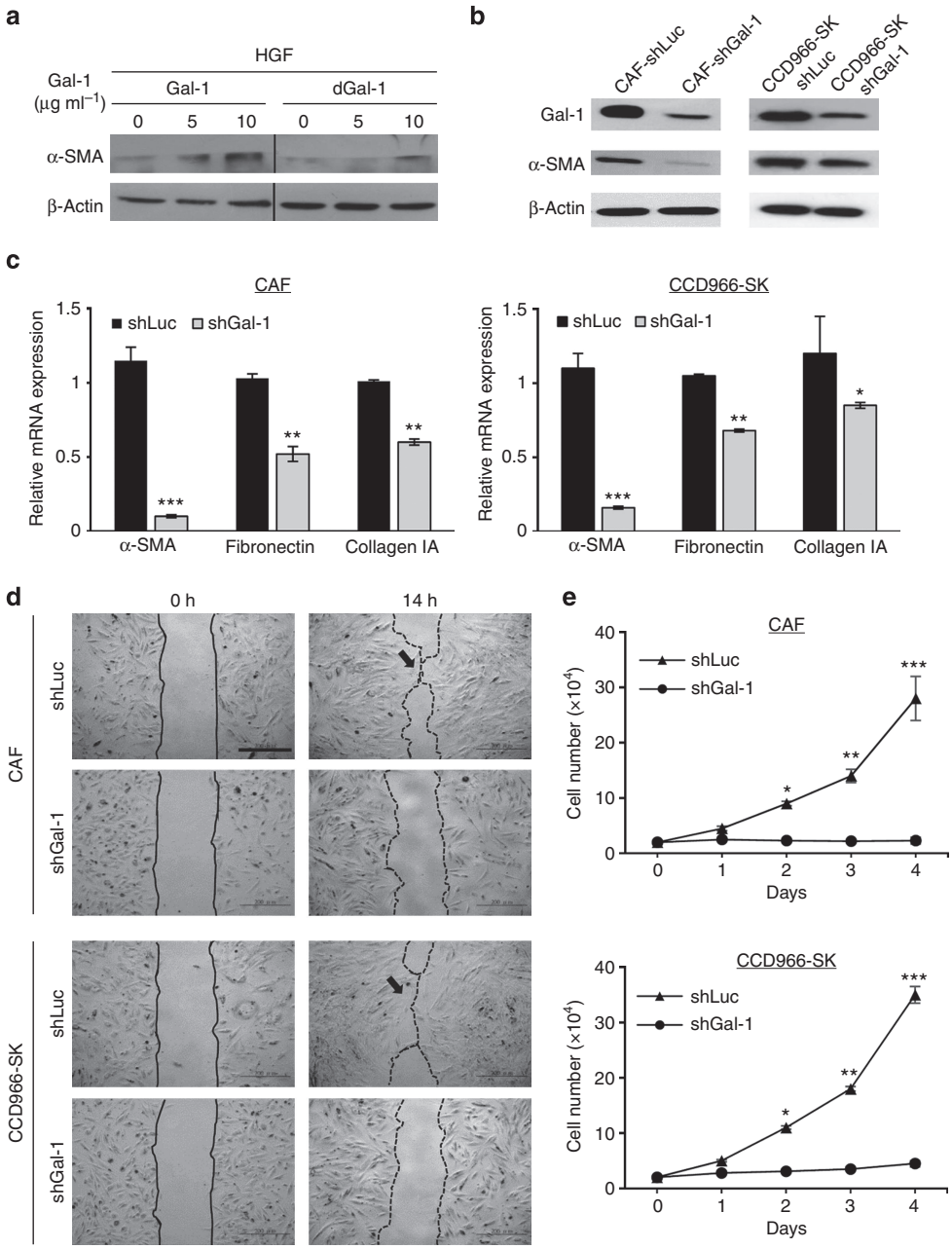

Figure 2. Galectin-1 promotes fibroblast activation, migration, and proliferation. (**a**) Western blotting of α-smooth muscle actin (α-SMA) in human gingival fibroblasts (HGFs) treated with various concentrations of active or heat-denatured galectin-1 for 24 hours. (**b**) Western blotting of α-SMA and Gal-1 in myofibroblasts. (**c**) The mRNA expression of α-SMA, fibronectin, and collagen IA in shGal-1-cells was compared with control cells (shLuc-cells, small hairpin RNA for luciferase). (**d**) A wound healing migration assay was described in the Materials and Methods. Full lines indicate the initial wound area and dotted lines indicate the migrating front of cells. Arrows indicate the cellular bridges. Scale bars = 200 μm. (**e**) Cell proliferation of shGal-1 and shLuc myofibroblasts was detected. Results are expressed as mean ± SEM, $n = 4$. *$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P \leqslant 0.001$.

signaling and activation of oral and skin myofibroblasts (Supplementary Figure S5c online). The Smad3/NOX4 pathway is known to mediate transforming growth factor-β1 (TGF-β1)-induced myofibroblast activation, but the relationship between Smad3 and NOX4 is still controversial (Cucoranu *et al.*, 2005; Hecker *et al.*, 2009). To investigate this relationship, HGFs were treated with SIS3, a specific phosphorylation inhibitor for Smad3, for 24 hours and then treated with galectin-1 before analysis of NOX4 and α-SMA expression. Inhibition of Smad3 effectively blocked galectin-1-induced NOX4 and α-SMA expression in fibroblast and myofibroblasts (Figure 5d and e), whereas phosphorylation of Smad3 was not affected in NOX4-depleted myofibroblasts (Supplementary Figure S5d online), indicating that NOX4 expression occurs downstream of Smad3. Previous studies have shown that NRP1 interacts with TGF-β1 during

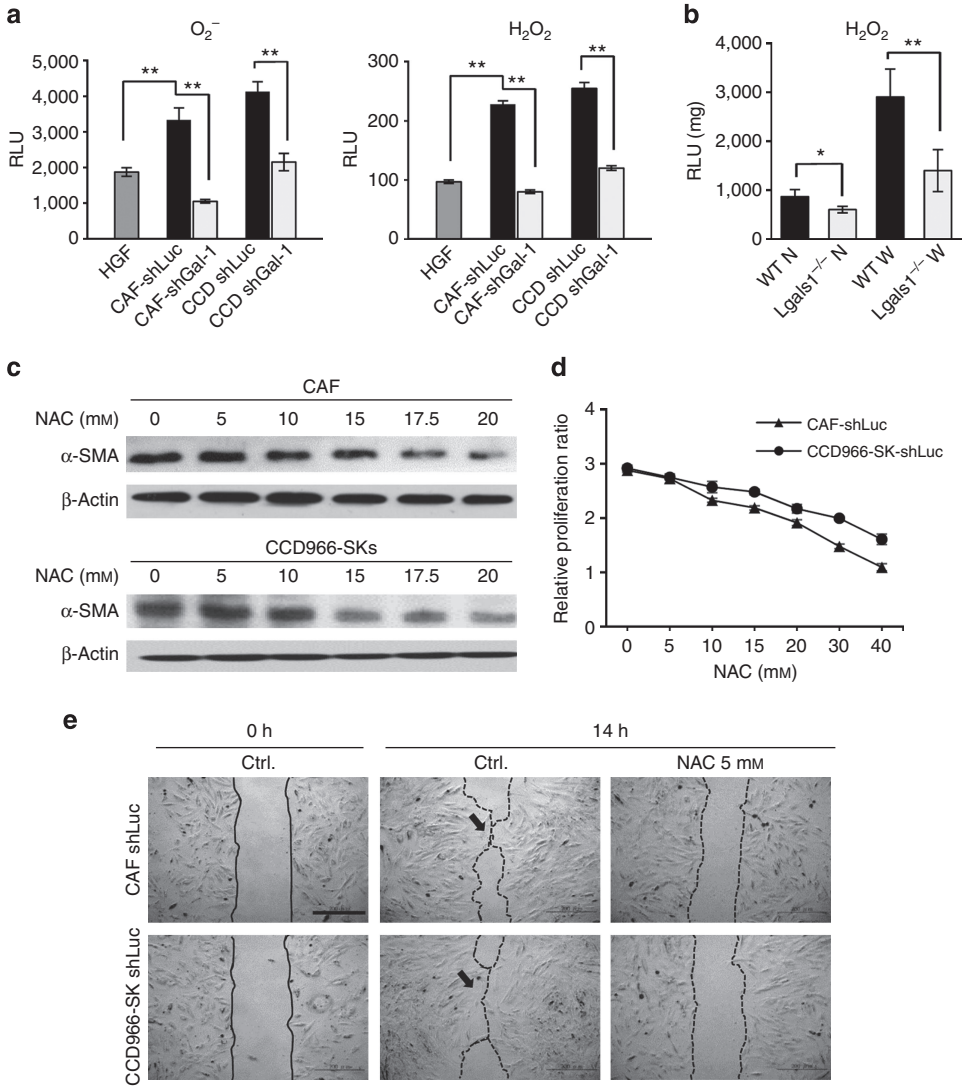

Figure 3. Galectin-1 triggers fibroblast activation by inducing reactive oxygen species (ROS) production *in vitro* and *in vivo*. (**a**) Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) production was detected by lucigenin and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), respectively. (**b**) H_2O_2 was detected in the sonicated extracts of normal and wound skin from wild-type (WT) and *LGALS1*$^{-/-}$ mice. (**c**–**e**) *N*-acetyl-L-cysteine (NAC) decreased α-smooth muscle actin (α-SMA) expression, cell proliferation, and migration in a concentration-dependent manner. Representative images of wound healing migration assay at 0 and 14 hours after NAC treatment. Intact lines indicate the initial wound area, dotted lines indicate the migrating front of cells, and arrows indicate the cellular bridges that formed in control cells. Scale bars = 200 μm. Results are expressed as mean ± SD, $n = 3$ per group. *$P \leqslant 0.05$; **$P \leqslant 0.01$.

myofibroblast activation. To test the possibility that galectin-1-induced Smad3 activation occurs through increased TGF-β signaling, HGFs were pretreated with SB-431542, an inhibitor of the type-I TGF-β receptor. No difference was observed upon treatment with SB-431542 (Supplementary Figure S5e online), suggesting that TGF-β signaling is not required for galectin-1-induced Smad3 phosphorylation.

Smad3/NOX4-mediated, galectin-1-induced wound healing was then demonstrated *in vivo*. Upregulation of galectin-1 was accompanied by increases in the levels of p-Smad3, total Smad3, and NOX4 in wounded tissues of WT mice (Figure 5f and Supplementary Figure S6 online), whereas only total Smad3 was upregulated in the wound tissues of *Lgals1*$^{-/-}$ mice. The expression levels of NRP1 and TGF-β1 in the wounds of WT and *Lgals1*$^{-/-}$ mice were not significantly different (Supplementary Figure S5f online). Smad3/NOX4-mediated, galectin-1-induced fibroblast activation was also demonstrated in murine dermal myofibroblasts (Figure 5g). Taken together, these results indicate that NRP1 acts as a receptor for galectin-1 in fibroblasts and mediates galectin-1-induced fibroblast activation during wound healing.

Galectin-1 accelerates normal and diabetic wound healing in animal models

We showed that galectin-1 induces signaling via the Smad3/NOX4 pathway and promotes ROS generation and that *Lgals1*$^{-/-}$ mice present delayed wound healing. Therefore, we evaluated whether topical administration of galectin-1 could augment wound closure in an animal model. Paired,

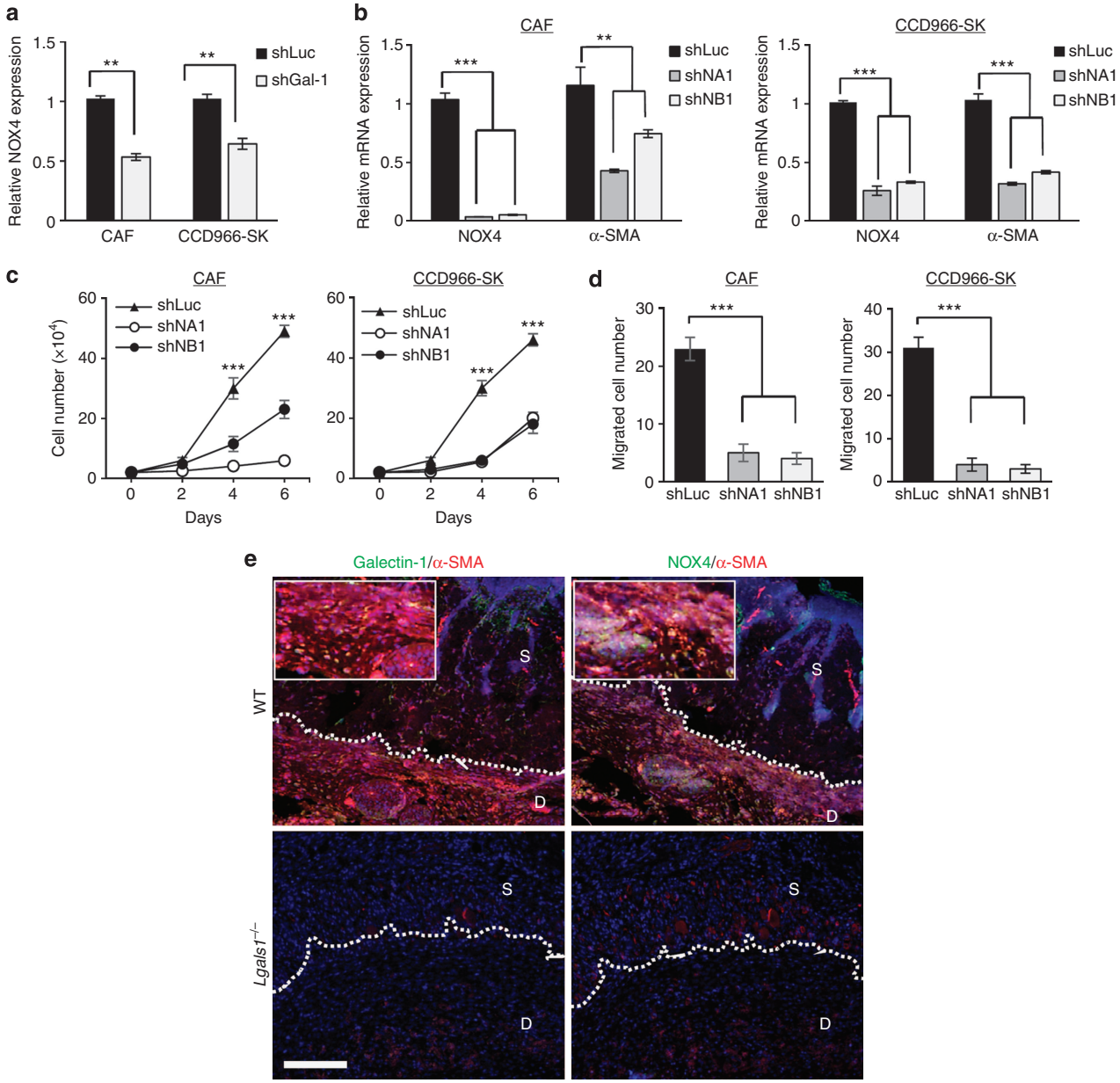

Figure 4. Galectin-1-induced reactive oxygen species (ROS) production and myofibroblast activation is NADPH oxidase 4 (NOX4) dependent. (**a**) The level of NOX4 mRNA in shLuc- and shGal-1 myofibroblasts was measured by quantitative real-time PCR (qRT-PCR). (**b–d**) Knockdown of NOX4 in myofibroblasts significantly decreased α-smooth muscle actin (α-SMA) expression, cell proliferation, and migration. (**e**) Representative immunofluorescent images of galectin-1/α-SMA and α-SMA/NOX4 in wound areas of wild-type (WT) and *Lgals1*$^{-/-}$ mice. Left panel: green, galectin-1; red, α-SMA. Right panel: green, NOX4; red, α-SMA; blue, 4′,6-diamidino-2-phenylindole (DAPI); yellow, colocalized cells. White dotted lines indicate the wound edge. White bars = 100 μm. Results are expressed as mean ± SEM, $n = 3$; $^{**}P \leqslant 0.01$; $^{***}P \leqslant 0.001$.

6-mm dorsal full-thickness excisional wounds were created in mice. At 3 days after the wounds were established, we topically injected 100 μg of recombinant human galectin-1 underneath the scar and measured the wound healing area. We found that wounds exposed to galectin-1 healed more rapidly than those treated with the control solution (Figure 6a and b).

To further investigate the effect of galectin-1 treatment on impaired wound healing, streptozotocin-induced diabetes mellitus mice were established (Rossini *et al.*, 1977). After 2 weeks, the blood glucose levels of these mice were all higher than 400 mg dl^{-1} (Supplementary Table S1 online). Wounds were generated by 6-mm dermal biopsy punches, and galectin-1 (80–100 μg per wound) was injected underneath the scar for 7 consecutive days. Wound healing was measured for 12 consecutive days. We found that wounds treated with galectin-1 healed significantly faster than those treated with phosphate-buffered saline after day 3 and severe illness and death were prevented in mice (Figure 6c and d, and Supplementary Figure S7a online). The body weight of control

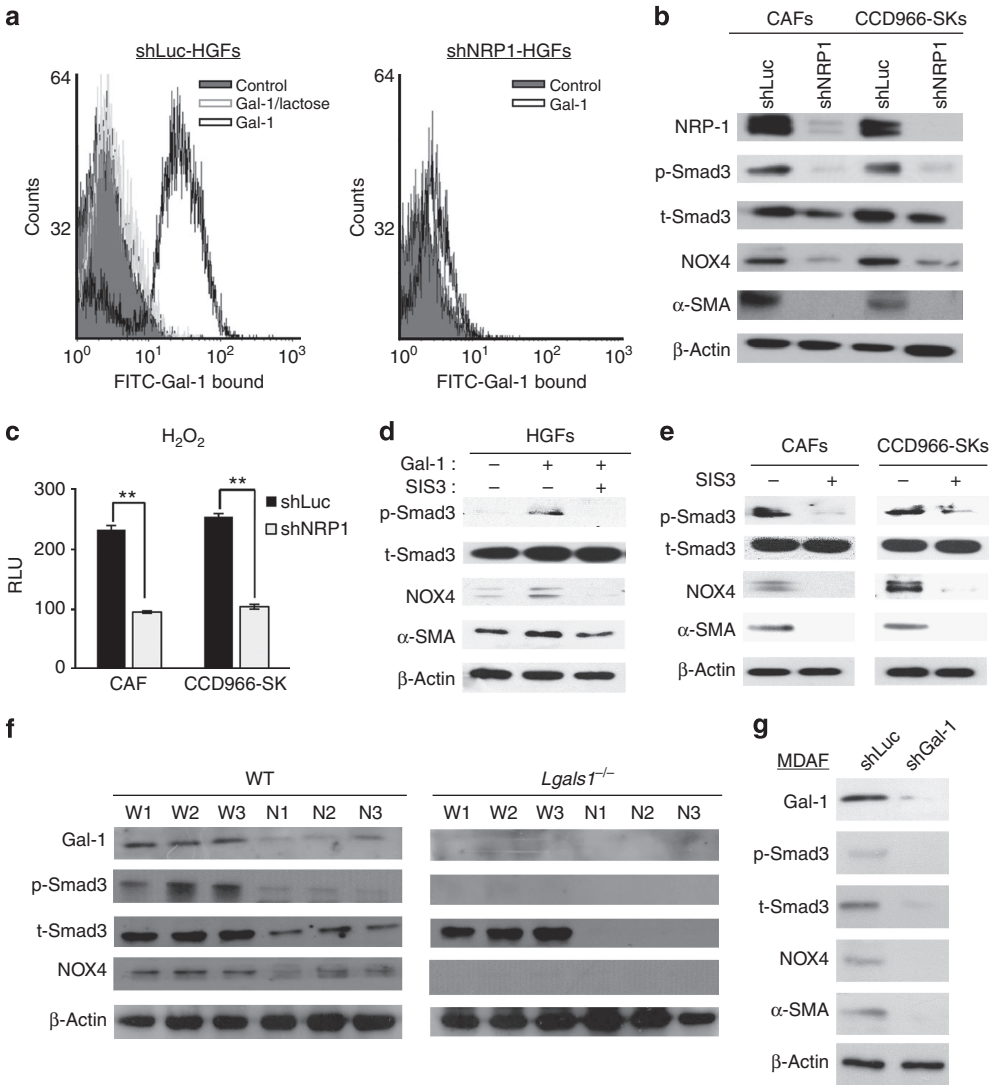

Figure 5. Galectin-1 activates fibroblasts by binding neuropilin-1 (NRP1) and regulating Smad3/NADPH oxidase 4 (NOX4) signaling. (**a**) Binding of FITC-galectin-1 to human gingival fibroblasts (HGFs) was analyzed. Left, closed gray curve, shLuc-HGFs only; open black curve, shLuc-HGFs with 20 μg ml^{-1} FITC-galectin-1; open gray curve, shLuc-HGFs with FITC-galectin-1 in the presence of lactose (20 mM). Right, closed gray curve, shNRP1-HGFs only; open black curve, shNRP1-HGFs with 20 μg ml^{-1} FITC-galectin-1. (**b**, **c**) Knockdown of NRP1 in myofibroblasts inhibited the Smad3/NOX4/α-smooth muscle actin (α-SMA) pathway and reactive oxygen species (ROS) production. (**d**, **e**) The effect of SIS3 on the expression of p-Smad3, t-Smad3, NOX4, and α-SMA in fibroblasts. (**f**, **g**) Western blotting analysis on the galectin-1/Smad3/NOX4 pathway in mouse wounds and murine dermal myofibroblasts (MDAFs). Results are expressed as mean ± SD, $n = 3$. $^{**}P \leqslant 0.01$.

mice was significantly decreased at day 8 (Supplementary Figure S7b online). In contrast, galectin-1-treated mice healed faster and survived well until 2 months, even though they still presented high blood glucose levels (Supplementary Figure S7c online). This observation suggests that galectin-1 not only reverses impaired wound healing in diabetic mice but also prevents severe illness and death. Therefore, galectin-1 treatment could be a potential therapy for impaired wound healing.

DISCUSSION

Elucidation of complex wound healing pathways is important for identifying therapeutic targets for clinical treatment. In this study, we showed that galectin-1 has an important role in myofibroblast activation and wound healing (Figure 6e). Murine skin wound healing experiments confirmed that reduced myofibroblast recruitment to wound sites in *Lgals1*$^{-/-}$ mice resulted in delayed wound healing. We also demonstrated that galectin-1 binds to NRP1 on myofibroblasts to activate Smad3 signaling and increase NOX4 expression and ROS production. Furthermore, topical administration of galectin-1 effectively augmented wound closure in normal and diabetic dermal excisional wound models. Evidence from this study strongly suggests that targeting of the galectin-1/NRP1/Smad3 pathway may be important for clinical therapy of general, severe, and disease (diabetes mellitus)-induced impaired wound healing.

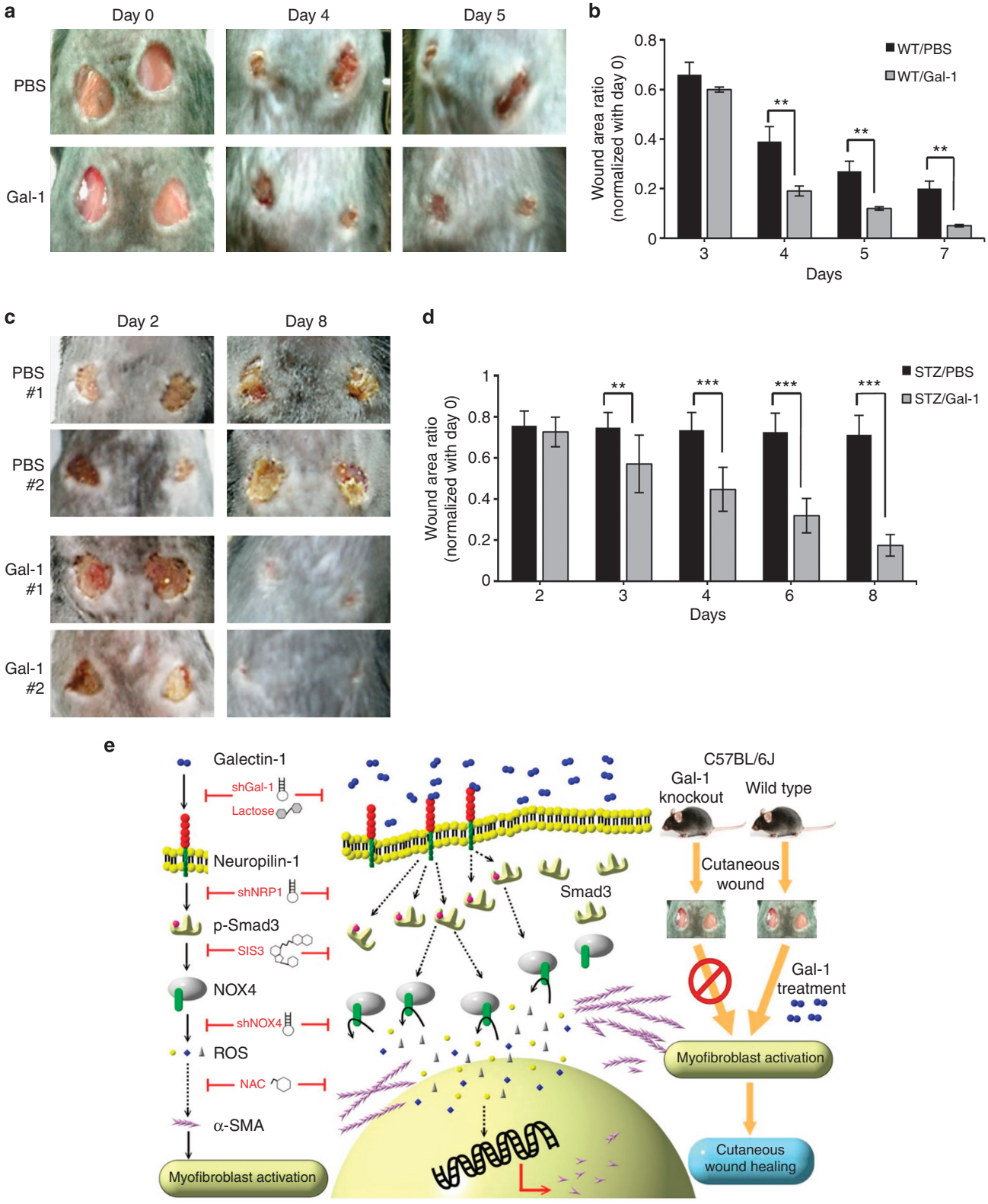

Figure 6. Topical administration of galectin-1 accelerates wound healing in animal models. (**a**) Representative macroscopic images of wounds in phosphate-buffered saline (PBS)- or galectin-1-treated healthy mice at 0, 4, and 5 days after wound show accelerated healing in galectin-1-treated wounds. (**b**) The area of the wounds was quantified by ImageJ software over multiple time points. (**c**, **d**) Images of wounds in PBS- or galectin-1-treated streptozotocin (STZ)-induced diabetes mellitus (DM) mice. Wound area was measured at the indicated time points and quantified by ImageJ analysis. (**e**) Diagram shows a working model of how galectin-1 promotes wound healing in mice with excisional wound through regulation of neuropilin-1 (NRP1)/Smad3/NADPH oxidase 4 (NOX4) signaling to modulate reactive oxygen species (ROS) production in myofibroblasts. Results are expressed as mean ± SD. $^{**}P \leqslant 0.01$; $^{***}P \leqslant 0.001$.

Galectin-1 is well known to be involved in development, differentiation, morphogenesis, tumor metastasis, and immune regulatory function (Smetana *et al.,* 2013). In this study, we demonstrated for the first time that galectin-1 can promote wound healing by inducing Smad3/NOX4 signaling to increase ROS production in myofibroblasts. However, the healing process is complicated, and different cells are involved in different phases; thus, galectin-1-mediated regulation of different cell types and healing phases during wound repair remains unclear. Recent immunohistochemical observations showed that the regulation of galectins during wound healing in rat skin and trachea is dynamic; galectin-1 is upregulated during the early phases of healing, and then galectin-3 is slightly upregulated during the later phases (Maeda *et al.,* 2004; Grendel *et al.*, 2012). In addition to its expression in myofibroblasts, galectin-1 is expressed in skin keratinocytes and mediates cell matrix interactions, suggesting a potential role in reepithelialization during wound healing (Chen *et al.*, 2012). However, a previous study showed that both endogenous galectin-1 and exogenously added galectin-1 did not affect the reepithelialization rate of corneal wounds (Cao *et al.*, 2002). In *Lgals1*$^{-/-}$ mice, galectin-1 has been demonstrated to have essential roles in the control of inflammation and neovascularization (Thijssen *et al.*, 2006; Seropian *et al.*, 2013). The effects of galectin-1 in other cell types, such as macrophages, neutrophils, and endothelial cells, during different healing phases should be studied further.

ROS have pivotal roles in physiological and pathological processes, including the progression of wound healing (auf dem Keller *et al.,* 2006; Bedard and Krause, 2007; Huo *et al.,* 2009; Bryan *et al.,* 2012). During wound healing, ROS production in constructive and destructive cells is associated with a fine balance in the remodeling of tissue. ROS are thought to act as cellular messengers to stimulate key processes associated with wound healing, including cell motility, cytokine activity, and angiogenesis. Here, we report that increased ROS production is required for galectin-1-regulated myofibroblast activation. The levels of ROS in skin wound areas in *Lgals1*$^{-/-}$ mice were significantly lower than those in WT mice, suggesting that ROS are involved in the effects of galectin-1 on wound repair. Maintaining the balance between oxidants and antioxidants is crucial for proper wound healing. Several investigators have studied the effects of growth factors (e.g., epidermal growth factor and TGF-β) on oxidative events during wound healing (Huo *et al.,* 2009; Bondi *et al.,* 2010). Recently, platelet-derived growth factor (PDGF)-BB, an active ingredient of the FDA (Food and Drug Administration)-approved wound treatment gel becaplermin that accelerates wound closure of chronic diabetic ulcers, was shown to significantly increase ROS levels in the early phase of wound healing, followed by a decrease in the late phase, suggesting that PDGF administration may modulate reactive oxidation depending on the stage of the wound healing process (Kaltalioglu *et al.,* 2013). Further studies are necessary to characterize the effects of exogenous galectin-1 administration or endogenous galectin-1 expression on reactive oxidation over the course of cutaneous wound healing, particularly to determine whether galectin-1 protects the oxidative balance.

NRP1 is a co-receptor for several growth factors and has multiple functions involved in development, immunity, and cancer. Although NRP1 was initially shown to bind semaphorin 3A and vascular endothelial growth factor to regulate axonal guidance and angiogenesis, recent findings have revealed that it has a much broader spectrum of activity. In particular, NRP1 binds TGF-β1, hepatocyte growth factor, and PDGF and their receptors (Prud'homme and Glinka, 2012). These ligands and pathways are all relevant to wound healing. Our previous study showed that NRP1 is a receptor for galectin-1 in vascular endothelial cells that promotes galectin-1-induced vascular endothelial growth factor receptor-2 signaling and endothelial migration (Hsieh *et al.,* 2008). In this study, we demonstrated that NRP1 is a predominant binding protein for galectin-1 in myofibroblasts and mediates galectin-1-induced myofibroblast activation. Depletion of NRP1 in myofibroblasts blocks galectin-1 binding and the regulation of Smad3/NOX4. An NRP1-neutralizing antibody was shown to ameliorate the recruitment of activated hepatic stellate cells that exhibit the highly motile myofibroblast phenotype, thereby blocking rat liver fibrosis induced by liver injury (Cao *et al.,* 2010a). Cao *et al.* (2010b) reported that elimination of NRP1 in fibroblasts decreased Smad2/3 phosphorylation and α-SMA expression and also arrested cell growth. These studies indicate that NRP1 might be a key mediator in maintaining tissue homeostasis.

Our results show that pretreatment with an inhibitor of the TGF-β1 receptor does not influence galectin-1-induced Smad3 activation or NOX4/ROS production. The levels of TGF-β1 in wounds were not different between WT and *Lgals1*$^{-/-}$ mice, but *Lgals1*$^{-/-}$ mice presented delayed wound repair, suggesting that augmentation of wound healing by galectin-1 is independent of TGF-β signaling. Conversely, we did not observe a change in galectin-1 expression upon TGF-β1 treatment (Wu *et al.,* 2011) that excludes the possibility that galectin-1 is the downstream mediator of TGF-β1-induced myofibroblast transdifferentiation. NRP1 has been shown to colocalize with PDGF-receptor β in hepatic stellate cells and increase PDGF binding to PDGF receptor β to promote downstream signaling and hepatic stellate cell activation (Cao *et al.,* 2010a). NOX4 is also critical for modulation of the pulmonary myofibroblast response to PDGF (Amara *et al.,* 2010). PDGF-induced myofibroblast transdifferentiation is associated with upregulation of galectin-1 (Fitzner *et al.,* 2005). Therefore, binding of galectin-1 to the NRP1/PDGFR receptor complex and its interaction with PDGF to regulate wound healing deserve further investigation.

In summary, our study demonstrated the important role and mechanism of action of galectin-1 in the course of wound healing *in vitro* and *in vivo*. Tuning of the galectin-1/NRP1/Smad3 signaling pathway may provide a therapeutic option for impaired wound healing. Consequently, analysis of clinical specimens based on this concept is warranted.

MATERIALS AND METHODS

Mice and cutaneous wound model

Lgals1$^{-/-}$ mice were originally produced and deposited in the Mutant Mouse Regional Resource Center by the Consortium for

Functional Glycomics supported by the National Institute of General Medical Sciences (GM62116, Poirier and Robertson, 1993) and were maintained in the National Cheng Kung University laboratory animal center. The animals were raised and cared for according to the guidelines of the National Science Council, Taiwan. The mouse experiments were approved by the institutional animal care and use committee. For further information, see Supplementary Materials and Methods online.

Isolation and culture of fibroblasts

Cancer-associated fibroblasts and HGFs were isolated from oral cancer tissues and normal human gingival tissues as previously described (Wu *et al.,* 2011). For further information, see Supplementary Materials and Methods online.

Quantitative real-time PCR

Primer sequences and other experiment details are reported in Supplementary Materials and Methods online.

Histochemical studies and immunohistochemistry

Serial 5-μm-thick histological sections were stained with hematoxylin and eosin or Masson's trichrome. Other details are reported in Supplementary Materials and Methods online.

Quantification of superoxide and hydrogen peroxide

Lucigenin-enhanced chemiluminescence and 2′,7′-dichlorodihydrofluorescein diacetate were used to measure O_2^- and H_2O_2, respectively. For further information, see Supplementary Materials and Methods online.

Statistical analysis

The experiments were assessed using unpaired Student's *t*-test or one-way analysis of variance. The values represent the mean ± SEM or SD. Prism 5 software (USA) was used for statistical analysis.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We acknowledge the NIH-sponsored Mutant Mouse Regional Resource Center (MMRRC) National System as the source of genetically altered mice for use in this study (*Lgals1*$^{-/-}$). We are grateful to the RNAi Core Lab, Research Center of Clinical Medicine, National Cheng Kung University Hospital, for providing services. RNAi reagents were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taiwan. This work was supported by grants from the National Science Council, Taiwan (NSC 101-2320-B-006-026-MY3, NSC102-2314-B-006-024-MY3, and NSC 102-2325-B-006-016).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Al-Salam S, Hashmi S (2014) Galectin-1 in early acute myocardial infarction. *PLoS One* 9:e86994

Almkvist J, Dahlgren C, Leffler H *et al.* (2002) Activation of the neutrophil nicotinamide adenine dinucleotide phosphate oxidase by galectin-1. *J Immunol* 168:4034–41

Amara N, Goven D, Prost F *et al.* (2010) NOX4/NADPH oxidase expression is increased in pulmonary fibroblasts from patients with idiopathic pulmonary fibrosis and mediates TGFbeta1-induced fibroblast differentiation into myofibroblasts. *Thorax* 65:733–8

auf dem Keller U, Kümin A, Braun S *et al.* (2006) Reactive oxygen species and their detoxification in healing skin wounds. *J Investig Dermatol Symp Proc* 11:106–11

Bedard K, Krause KH (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 87:245–313

Bondi CD, Manickam N, Lee DY *et al.* (2010) NAD(P)H oxidase mediates TGF-beta1-induced activation of kidney myofibroblasts. *J Am Soc Nephrol* 21:93–102

Bryan N, Ahswin H, Smart N *et al.* (2012) Reactive oxygen species (ROS)–a family of fate deciding molecules pivotal in constructive inflammation and wound healing. *Eur Cell Mater* 24:249–65

Cao S, Yaqoob U, Das A *et al.* (2010a) Neuropilin-1 promotes cirrhosis of the rodent and human liver by enhancing PDGF/TGF-beta signaling in hepatic stellate cells. *J Clin Invest* 120:2379–94

Cao Y, Szabolcs A, Dutta SK *et al.* (2010b) Neuropilin-1 mediates divergent R-Smad signaling and the myofibroblast phenotype. *J Biol Chem* 285: 31840–8

Cao Z, Said N, Amin S *et al.* (2002) Galectins-3 and -7, but not galectin-1, play a role in re-epithelialization of wounds. *J Biol Chem* 277:42299–305

Chen HY, Lo CH, Li CS *et al.* (2012) Galectins and cutaneous immunity. *Dermatol Sinica* 30:121–7

Clark RA (2008) Oxidative stress and "senescent" fibroblasts in non-healing wounds as potential therapeutic targets. *J Invest Dermatol* 128:2361–4

Clark RA, Ghosh K, Tonnesen MG (2007) Tissue engineering for cutaneous wounds. *J Invest Dermatol* 127:1018–29

Cucoranu I, Clempus R, Dikalova A *et al.* (2005) NAD(P)H oxidase 4 mediates transforming growth factor-beta1-induced differentiation of cardiac fibroblasts into myofibroblasts. *Circ Res* 97:900–7

Darby I, Skalli O, Gabbiani G (1990) Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Lab Invest* 63:21–9

Diegelmann RF, Evans MC (2004) Wound healing: an overview of acute, fibrotic and delayed healing. *Front Biosci* 9:283–9

Dvořánková B, Szabo P, Lacina L *et al.* (2011) Human galectins induce conversion of dermal fibroblasts into myofibroblasts and production of extracellular matrix: potential application in tissue engineering and wound repair. *Cells Tissues Organs* 194:469–80

Fitzner B, Walzel H, Sparmann G *et al.* (2005) Galectin-1 is an inductor of pancreatic stellate cell activation. *Cell Signal* 17:1240–7

Gál P, Vasilenko T, Kostelnikova M *et al.* (2011) Open wound healing in vivo: monitoring binding and presence of adhesion/growth-regulatory galectins in rat skin during the course of complete re-epithelialization. *Acta Histochem Cytochem* 44:191–9

Goldring K, Jones GE, Thiagarajah R *et al.* (2002) The effect of galectin-1 on the differentiation of fibroblasts and myoblasts in vitro. *J Cell Sci* 115: 355–66

Graves DT, Liu R, Alikhani M *et al.* (2006) Diabetes-enhanced inflammation and apoptosis–impact on periodontal pathology. *J Dent Res* 85:15–21

Grendel T, Sokolsky J, Vascakova A *et al.* (2012) Early stages of trachea healing process: (immuno/lectin) histochemical monitoring of selected markers and adhesion/growth-regulatory endogenous lectins. *Folia Biol (Praha)* 58:135–43

Guo S, Dipietro LA (2010) Factors affecting wound healing. *J Dent Res* 89:219–29

Harding KG, Moore K, Phillips TJ (2005) Wound chronicity and fibroblast senescence–implications for treatment. *Int Wound J* 2:364–8

Hecker L, Vittal R, Jones T *et al.* (2009) NADPH oxidase-4 mediates myofibroblast activation and fibrogenic responses to lung injury. *Nat Med* 15:1077–81

Hsieh SH, Ying NW, Wu MH *et al.* (2008) Galectin-1, a novel ligand of neuropilin-1, activates VEGFR-2 signaling and modulates the migration of vascular endothelial cells. *Oncogene* 27:3746–53

Huo Y, Qiu WY, Pan Q *et al.* (2009) Reactive oxygen species (ROS) are essential mediators in epidermal growth factor (EGF)-stimulated corneal

epithelial cell proliferation, adhesion, migration, and wound healing. *Exp Eye Res* 89:876–86

Kaltalioglu K, Coskun-Cevher S, Tugcu-Demiroz F *et al.* (2013) PDGF supplementation alters oxidative events in wound healing process: a time course study. *Arch Dermatol Res* 305:415–22

Klíma J, Lacina L, Dvorankova B *et al.* (2009) Differential regulation of galectin expression/reactivity during wound healing in porcine skin and in cultures of epidermal cells with functional impact on migration. *Physiol Res* 58:873–84

Lai A, Hosseini-Tabatabaei A, Hartwell R *et al.* (2013) Topical application of aminopeptidase N-neutralizing antibody accelerates wound closure. *Mol Cell Biochem* 372:95–100

Lerman OZ, Galiano RD, Armour M *et al.* (2003) Cellular dysfunction in the diabetic fibroblast: impairment in migration, vascular endothelial growth factor production, and response to hypoxia. *Am J Pathol* 162:303–12

Loots MA, Lamme EN, Mekkes JR *et al.* (1999) Cultured fibroblasts from chronic diabetic wounds on the lower extremity (non-insulin-dependent diabetes mellitus) show disturbed proliferation. *Arch Dermatol Res* 291:93–9

Maeda N, Kawada N, Seki S *et al.* (2003) Stimulation of proliferation of rat hepatic stellate cells by galectin-1 and galectin-3 through different intracellular signaling pathways. *J Biol Chem* 278:18938–44

Maeda N, Kawada N, Seki S *et al.* (2004) Involvement of Galectin-1 and Galectin-3 in proliferation and migration of rat hepatic stellate cells in culture. *Comp Hepatol* 3:S10

Masamune A, Satoh M, Hirabayashi J *et al.* (2006) Galectin-1 induces chemokine production and proliferation in pancreatic stellate cells. *Am J Physiol Gastrointest Liver Physiol* 290:G729–36

Menke NB, Ward KR, Witten TM *et al.* (2007) Impaired wound healing. *Clin Dermatol* 25:19–25

Poirier F, Robertson EJ (1993) Normal development of mice carrying a null mutation in the gene encoding the L14 S-type lectin. *Development* 119:1229–36

Prud'homme GJ, Glinka Y (2012) Neuropilins are multifunctional coreceptors involved in tumor initiation, growth, metastasis and immunity. *Oncotarget* 3:921–39

Rossini AA, Like AA, Chick WL *et al.* (1977) Studies of streptozotocin- induced insulitis and diabetes. *Proc Natl Acad Sci USA* 74:2485–9

Sakaguchi M, Arruda-Carvalho M, Kang NH *et al.* (2011) Impaired spatial and contextual memory formation in galectin-1 deficient mice. *Mol Brain* 4:33

Salatino M, Croci DO, Bianco GA *et al.* (2008) Galectin-1 as a potential therapeutic target in autoimmune disorders and cancer. *Expert Opin Biol Ther* 8:45–57

Sampson N, Berger P, Zenzmaier C (2012) Therapeutic targeting of redox signaling in myofibroblast differentiation and age-related fibrotic disease. *Oxid Med Cell Longev* 2012:458276

Sampson N, Koziel R, Zenzmaier C *et al.* (2011) ROS signaling by NOX4 drives fibroblast-to-myofibroblast differentiation in the diseased prostatic stroma. *Mol Endocrinol* 25:503–15

Sarrazy V, Billet F, Micallef L *et al.* (2011) Mechanisms of pathological scarring: role of myofibroblasts and current developments. *Wound Repair Regen* 19:s10–5

Seropian IM, Cerliani JP, Toldo S *et al.* (2013) Galectin-1 controls cardiac inflammation and ventricular remodeling during acute myocardial infarction. *Am J Pathol* 182:29–40

Singer AJ, Clark RA (1999) Cutaneous wound healing. *N Engl J Med* 341: 738–46

Smetana K Jr, André S, Kaltner H *et al.* (2013) Context-dependent multifunctionality of galectin-1: a challenge for defining the lectin as therapeutic target. *Expert Opin Ther Targets* 17:379–92

Thijssen VL, Postel R, Brandwijk RJ *et al.* (2006) Galectin-1 is essential in tumor angiogenesis and is a target for antiangiogenesis therapy. *Proc Natl Acad Sci USA* 103:15975–80

Tomasek JJ, Gabbiani G, Hinz B *et al.* (2002) Myofibroblasts and mechanoregulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 3: 349–63

Velnar T, Bailey T, Smrkolj V (2009) The wound healing process: an overview of the cellular and molecular mechanisms. *J Int Med Res* 37:1528–42

Welt K, Hinrichs R, Weiss JM *et al.* (2009) Skin diseases in Europe. Wound healing. *Eur J Dermatol* 19:413–6

Wu MH, Hong HC, Hong TM *et al.* (2011) Targeting galectin-1 in carcinoma-associated fibroblasts inhibits oral squamous cell carcinoma metastasis by downregulating MCP-1/CCL2 expression. *Clin Cancer Res* 17:1306–16

Wu MH, Hong TM, Cheng HW *et al.* (2009) Galectin-1-mediated tumor invasion and metastasis, up-regulated matrix metalloproteinase expression, and reorganized actin cytoskeletons. *Mol Cancer Res* 7:311–8

Xue X, Lu Z, Tang D *et al.* (2011) Galectin-1 secreted by activated stellate cells in pancreatic ductal adenocarcinoma stroma promotes proliferation and invasion of pancreatic cancer cells: an in vitro study on the microenvironment of pancreatic ductal adenocarcinoma. *Pancreas* 40:832–9